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C1qTNF-related protein-6 mediates fatty acid oxidation via the activation of the AMP-activated protein kinase

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ABSTRACT

C1qTNF-related proteins (CTRPs) are involved in diverse processes including metabolism, inflammation host defense, apoptosis, cell differentiation, autoimmunity, hibernation, and organogenesis. However, the physiological role of CTRP6 remains poorly understood. Here we demonstrate that the globular domain of CTRP6 mediates the phosphorylation and activation of the 5′-AMP-activated protein kinase (AMPK) in skeletal muscle cells. In parallel with the activation of AMPK, CTRP6 induces the phosphorylation of acetyl coenzyme A carboxylase (ACC) and fatty acid oxidation in myocytes. Thus, CTRP6 plays a role in fatty acid metabolism via the AMPK-ACC pathway.

1. Introduction

C1qTNF-related protein-6 (CTRP6) is a 240-amino acid protein that consists of a signal peptide, a short variable region, a collagen-like region, and a C-terminal globular domain. It belongs to a family of proteins characterized by a common TNF alpha-like globular domain. Although CTRP family proteins are structurally related, they are involved in diverse processes including metabolism, inflammation host defense, apoptosis, cell differentiation, autoimmunity, hibernation, and organogenesis [1,2]. CTRP1 inhibits collagen-induced platelet aggregation by specifically blocking binding of the von Willebrand factor to collagen [3] and also stimulates aldosterone production [4]. CTRP2 increases glycogen accumulation and fatty acid oxidation by the activation of AMP-activated protein kinase [5]. CTRP3/CORS26/Carducin is involved in regulating both chondrogenesis and cartilage development, and also has anti-inflammatory properties [6,7]. Mutation of CTRP5 leads to insufficient levels of secreted CTRP5, causing late-onset retinal macular degeneration [8,9]. CTRP9 reduces serum glucose and forms a heterodimer with adiponectin [10]. However, the function of CTRP6 remains poorly investigated.

AMP-activated protein kinase (AMPK) is a key regulatory enzyme that controls cellular and whole-body energy homeostasis [11]. AMPK is mainly activated by an increase in the AMP to ATP ratio, and it participates in cellular energy control by turning on ATP-generating catabolic pathways, such as glucose transport and fatty acid oxidation [11,12]. When activated by hypoxia or muscle contraction, AMPK induces phosphorylation of acetyl-CoA carboxylase (ACC), thereby activating fatty acid oxidation [13,14]. Recently, a new, highly conserved family of adiponectin paralogs, designated as CTRP isoforms 1–10, was identified by searching mouse and human ESTs and genome databases [5].

Among these, CTRP2 is similar to adiponectin and rapidly induces phosphorylation of AMPK, ACC, and mitogen-activated protein kinase (MAPK) in C2C12 myotubes, which increases glycogen accumulation and fatty acid oxidation [15]. We previously showed that CTRP5 activates AMPK and is increased in mitochondrial DNA (mtDNA)-depleted myocytes [10]. A recent study showed that the expression level of CTRP6 is significantly increased in 12-week *ob/ob* mice relative to lean controls [5]. These findings compelled us to speculate that CTRP6 may be involved in fat metabolism. In this study, we present evidence that CTRP6 can stimulate fatty acid oxidation via the activation of AMP-activated protein kinase (AMPK).

2. Materials and methods

2.1. Reagents

Polyclonal antibodies directed against AMPKα, phospho-AMPKα (Thr172), phospho-ACC (Ser79), Akt, phospho-Akt (Ser473),

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p38 MAPK, and phospho-p38 MAPK were obtained from Cell Signaling Technology. The anti-actin antibody was purchased from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz. AICAR was acquired from Toronto Research Chemicals. Insulin (porcine crystalline) and Adenine 9-β-D-arabinofuranoside (AraA) were acquired from Sigma.

2.2. Cell culture

C2C12 myocytes (ATCC CCL-1) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and the appropriate antibiotics. To induce differentiation, the cells were grown to 100% confluence in maintenance medium and then switched to differentiation medium (DMEM supplemented with 2% horse serum).

2.3. Generation of recombinant CTRP6 proteins

To produce GST-fusion protein corresponding to the globular domain of human CTRP-6, the fragment of CTRP-6 cDNA encoding amino acids 145-273 was generated by PCR and cloned into the *BamH*I and *Xho*I sites of pGEX 4T-1. The recombinant protein was expressed in *Escherichia coli* BL21, harvested, and purified using Glutathione Sepharose™ fast flow, in accordance with the manufacturer's instructions (Pharmacia). To remove endotoxin, the purified proteins were loaded in Detoxi-Gel (Pierce) before storage at −70 °C.

2.4. Phosphoprotein analysis

C2C12 myotubes were serum-starved for 2 h and treated with AICAR (2 mM), insulin (100 nM) or recombinant proteins. The cells were immediately washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in cold lysis buffer [20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM pyrophosphatase, 1 μM pepstatin A, 1 μM leupeptin, and 1 μM aprotinin] for 30 min with vortex mixing. Equal amounts (30 μg) of cell lysates were resolved by 6% or 10% SDS–PAGE, and separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membranes were incubated in blocking solution consisting of 5% skim milk in TTBS [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature, and then immunoblotted with the indicated phospho-specific antibody. After overnight incubation, the membranes were extensively washed with TTBS and incubated with HRP-conjugated anti-rabbit or anti-mouse IgG. Proteins were visualized using an enhanced chemiluminescent substrate kit (Amersham Bioscience). Immunoblot intensities were quantitated by densitometry using an analytical scanning system (Molecular Dynamics Inc., Sunnyvale, CA).

2.5. Fatty acid oxidation

Fatty acid oxidation assays were performed as previously described [16] with slight modifications. Briefly, C2C12 myotubes were serum-starved for 2 h and incubated with preincubation buffer (DMEM medium containing 4 mM glutamine, 25 mM HEPES, 1% fatty acid-free BSA, and 0.25 mM palmitate) for 1 h in 12-well plates. Following addition of ^{14}C-palmitate (1 μCi/ml), the cells were incubated for 90 min at 37 °C in the presence of globular CTRP6 (10 μg/ml) or GST (10 μg/ml) proteins. Each well was covered with a piece of Whatman paper and fixed using tape and incubated for 90 min at 37 °C. After incubation, 200 μl of 3 M NaOH solution was applied to the edge of the paper and saturated the entire sheet by capillary action, and then 0.1 ml of 70% perchloric acid (Sigma) was injected into the wells with a syringe. The filter paper was removed from each well after collection of CO_2 for 1 h at room temperature. The amount of ^{14}C-radioactivity was determined by a liquid scintillation counter (Perkin–Elmer). For some experiments, the cells were pretreated with AraA (2 mM) for 1 h prior to addition of gCTRP6.

2.6. Statistical analysis

Statistical significance was assessed using *t*-test and ANOVA. A *P* value of <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. The globular domain of CTRP6 stimulates the phosphorylation of AMPK in a dose- and time-dependent manner

Several CTRP isoforms, such as CTRP2, CTRP5, and CTRP9, induce phosphorylation of AMP-activated protein kinase (AMPK) in C2C12 myotubes, which thereby regulates glucose and fat metabolism [5,15,17]. The CTRP6 transcript is significantly upregulated in 12-week *ob/ob* mice relative to age-matched controls, suggesting that CTRP6 is potentially involved in fat metabolism [10]. To investigate the effect of CTRP6 on the activation of AMPK, which plays a central role in fat metabolism, we generated full-length and the globular domain of human CTRP6 as GST-fusion proteins. Although we made multiple efforts, the full-length CTRP6 could be produced only at very low concentrations. In a recent study, the size of serum CTRP6 on immunoblots corresponded to its globular head rather than the full-length protein [16]. We therefore used the globular domain of CTRP6 (gCTRP6) for our experiments. Treatment with gCTRP6 rapidly stimulated the phosphorylation of AMPK in C2C12 myotubes (Fig. 1A). The effect peaked at 45 min (3.5-fold increase over the untreated control; $P < 0.001$) (Fig. 1B). In addition, the effects of gCTRP6 on AMPK phosphorylation occurred in a dose-dependent manner (Fig. 1C). After treatment with 10 μg/ml of gCTRP6, AMPK phosphorylation increased by approximately four-fold, which is comparable to the effects of stimulation by AICAR, a cell-permeable activator of AMPK ($P = 0.004$) (Fig. 1D). GST alone had no effect on activation of AMPK. These results indicate that CTRP6 could be involved in fat metabolism via the activation of AMPK.

3.2. The AMPK-ACC pathway is a target of CTRP6

The activation of AMPK stimulates the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC), with a consequent reduction of malonyl-CoA synthesis that stimulates fatty acid oxidation [15]. Therefore, we investigated whether gCTRP6 induces the phosphorylation of ACC in C2C12 myotubes. In parallel with activation of AMPK, treatment of C2C12 myotubes with gCTRP6 induced the phosphorylation of ACC (Fig. 2A). After treatment with 10 μg/ml of gCTRP6, ACC phosphorylation increased by approximately 2.9-fold, compared to GST-treated control ($P = 0.001$) (Fig. 2B). This result suggests that gCTRP6 stimulates the deactivation of ACC through the activation of AMPK. In skeletal muscle, adiponectin activates p38 MAPK in addition to AMPK, which increases glucose uptake and fatty acid oxidation [18]. In a recent study, CTRP9 activates AMPK and Akt in C2C12 myotubes and lowered serum glucose levels in obese mice [19]. However, treatment of C2C12 myotubes with the globular domain of CTRP6 did not induce the phosphorylation of p38 MAPK or the insulin signaling intermediate, Akt (Fig. 2). Thus, CTRP6 is a potent and selective activator of the AMPK-ACC pathway and is independent of the

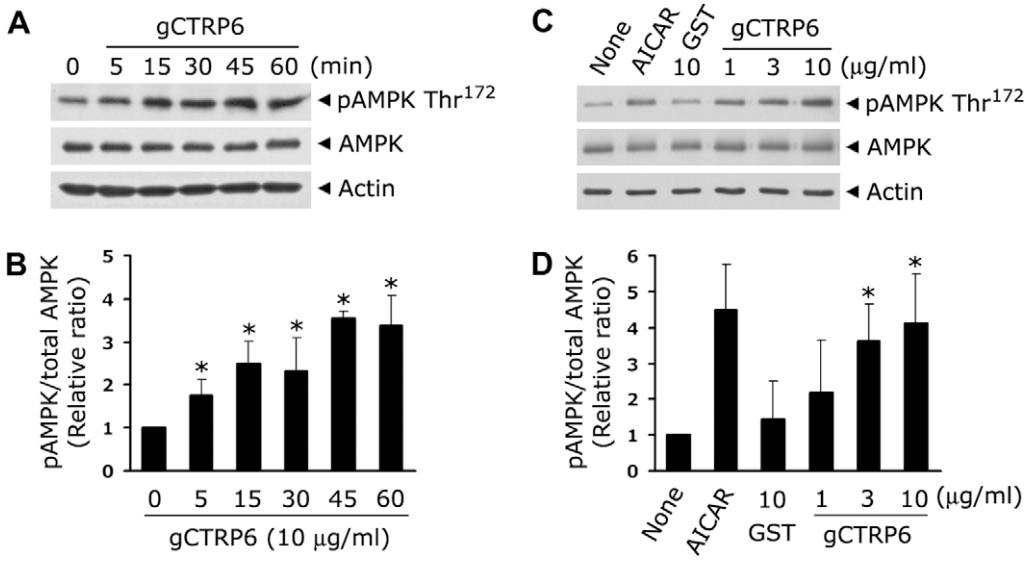

Fig. 1. Phosphorylation of AMPK by the globular domain of human CTRP6 in C2C12 myotubes. (A) C2C12 myotubes were treated with 10 μg/ml of GST-fused globular domain of human CTRP6 (gCTRP6) for the indicated times. The cells were lysed as described in Section 2. Equal amounts (30 μg) of cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies directed against AMPK, phospho-AMPK (Thr172, pAMPK), and actin. A representative result from three independent experiments is shown. (B) Immunoblot intensities for pAMPK/AMPK were quantitated by densitometry and expressed in arbitrary units. The intensity of the untreated control was set to one. Results are expressed as the mean ± S.D. of three independent experiments. *t*-test; $^{*}P < 0.05$ compared with untreated control. (C) C2C12 myotubes were serum-starved for 2 h and treated with AICAR (2 mM), GST (10 μg/ml), or one of three different concentrations (1, 3, and 10 μg/ml) of gCTRP6 for 45 min. Immunoblot analysis was performed using antibodies directed against AMPK, phospho-AMPK, and actin. A representative result from three independent experiments is shown. (D) Immunoblot intensities for pAMPK/AMPK were quantitated by densitometry and expressed in arbitrary units. The intensity of the untreated control was set to one. Results are expressed as the mean ± S.D. of three independent experiments. *t*-test; $^{*}P < 0.05$ compared with untreated control.

insulin signaling pathway. To further confirm the effect of CTRP6 on the activation of AMPK-ACC pathway, we examined the effect of AraA, an inhibitor of AMPK, on CTRP6-mediated signaling. Pretreatment with AraA significantly inhibited gCTRP6-stimulated phosphorylation of AMPK and ACC (Fig. 3), indicating that ACC is a downstream signaling molecule of CTRP6-stimulated phosphorylation of AMPK.

3.3. gCTRP6 induces fatty acid oxidation

In muscle, activated AMPK phosphorylates and inhibits ACC, leading to a decrease in fatty acid synthesis and a concomitant increase in the β-oxidation of fatty acids [20]. We investigated whether gCTRP6 stimulates fatty acid oxidation. Treatment with the globular domain of CTRP6 caused a 1.5-fold increase in ^{14}C-palmitate oxidation in C2C12 myotubes compared to untreated control ($P < 0.002$), whereas GST alone had no effect (Fig. 4A). In addition, gCTRP6-mediated palmitate oxidation was significantly inhibited by pretreatment of araA ($P = 0.004$) (Fig. 4B). Since CTRP6 phosphorylates ACC via the activation of AMPK, the induction of fatty acid oxidation by CTRP6 is mainly due to activation of the AMPK-ACC pathway.

CTRP family proteins all consist of an N-terminal signal peptide, a variable region, a collagen (Gly-X-Y) repeat and a C-terminal C1q-like globular domain [10]. The last exon of CTRP6 encodes the entire C-terminal globular domain, which is a functional domain that may interact with the protein's ligand or a receptor. A fragment corresponding to the globular domain of adiponectin (globular adiponectin) has been shown to be present in serum [5] and to exhibit potent metabolic effects [20–23]. Furthermore, globular adiponectin has a high affinity for binding to AdipoR1, which is predominantly expressed in skeletal muscle [24]. Since the size of CTRP6 in serum corresponds to its globular domain rather than the full-length protein [25], CTRP6 may undergo proteolytic processing. In this study, we found that the globular domain of CTRP6 mediates fatty acid oxidation via activation of the AMPK-ACC pathway. Among CTRP isoforms involved in glucose and fat metabolism, CTRP2 and CTRP9 are predominantly expressed in adipose tissues [26]. However, CTRP5 and CTRP6 are widely expressed in many tissues [15], suggesting that they may act in physiological or pathological conditions via a different expression mechanism. Indeed, we recently found that CTRP5 is upregulated in mtDNA-depleted myocytes and activates AMPK [15]. In a recent study, the serum level of CTRP6 was significantly increased in adiponectin-null mice [15]. Consistent with this finding, CTRP6 transcripts were increased in 12-week-old *ob/ob* mice, which showed decreased adiponectin expression relative to their lean controls [16]. Considering that adiponectin activates AMPK and fatty acid oxidation in human skeletal muscles [15], these findings raise the possibility that, under certain conditions, CTRP6 plays a role in compensating for the loss of adiponectin. In addition, CTRP1 is the closest homolog of CTRP6 among CTRP family proteins and can form a heterodimer with CTRP6 [15,27], which suggests that CTRP1 may also be involved in CTRP6 functions, such as fatty acid oxidation. It remains to be determined whether the two proteins are functionally linked.

Although our results provide evidence that CTRP6-stimulated phosphorylation of AMPK activates fatty acid oxidation, activation of AMPK also can induce an increase in glucose uptake via GLUT4 translocation [5]. It has been reported that globular adiponectin and CTRP5 induces glucose uptake via the translocation of GLUT4 [15,26]. Our preliminary data show that the globular domain of CTRP6 can also stimulate the translocation of GLUT4 in L6 GLUT4-myc cells (unpublished data). Thus, it remains to be determined whether CTRP6 induces glucose uptake via the translocation of GLUT4. In summary, the results of the present study show that CTRP6 mediates fatty acid oxidation via the AMPK-ACC pathway. Although the expression pattern and function of CTRP6 in metabolic diseases is still unclear, the identification of CTRP6 as a functional protein that mediates the oxidation of fatty acids in muscle

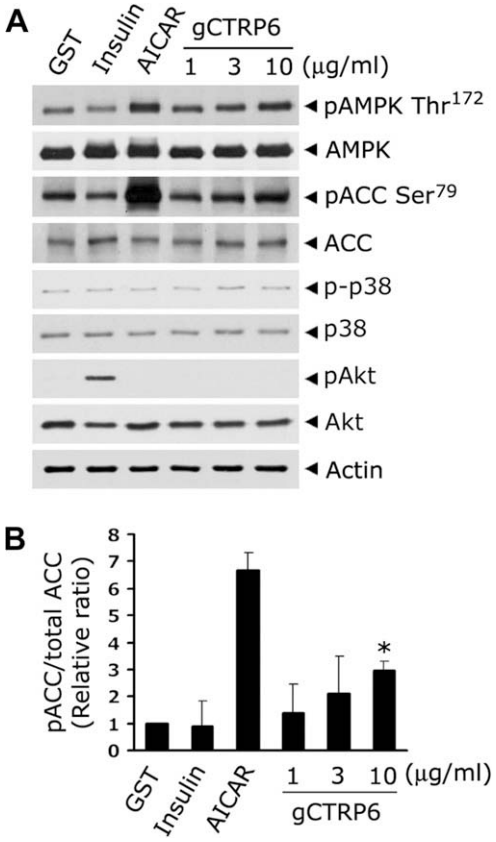

Fig. 2. gCTRP6 induces the phosphorylation of ACC and is independent of Akt and p38 MAPK pathways. (A) C2C12 myotubes were serum-starved for 2 h and treated with insulin (100 nM), AICAR (2 mM), GST (10 μg/ml), or three different concentrations (1, 3, and 10 μg/ml) of gCTRP6 for 45 min. The cells were lysed as described in Section 2. Equal amounts (30 μg) of cell lysates were subjected to SDS–PAGE and immunoblotted with antibodies directed against AMPK, phospho-AMPK (Thr172, pAMPK), ACC, phospho-ACC (Ser79, pACC), Akt, phospho-Akt (Ser473, pAkt), p38 MAPK (p38), phospho-p38 MAPK (pp38), and actin. A representative result from three independent experiments is shown. (B) Immunoblot intensities for pACC/ACC were quantitated by densitometry and expressed in arbitrary units. The intensity for GST-treated control was set to one. Results are expressed as the means ± S.D. of three independent experiments. *t*-test; $^{*}P < 0.05$ compared with GST control.

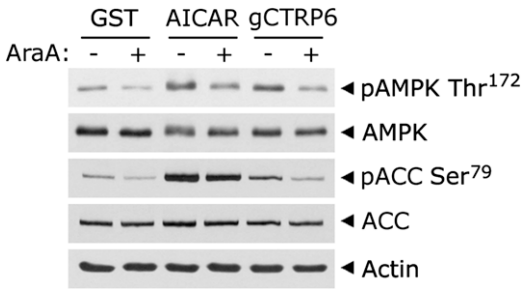

Fig. 3. AraA, an inhibitor of AMPK, inhibits the phosphorylation of AMPK and ACC by globular CTRP6. C2C12 myotubes were serum-starved for 2 h and treated with AICAR (2 mM), GST (10 μg/ml), or gCTRP6 (10 μg/ml), with or without 1-h pretreatment of AraA (2 mM). The cells were lysed as described in Section 2. Equal amounts (30 μg) of cell lysates were subjected to SDS–PAGE and immunoblotted with antibodies directed against AMPK, phospho-AMPK, ACC, phospho-ACC, and actin. A representative result from three independent experiments is shown.

cells may provide a novel therapeutic and diagnostic target for metabolic syndromes such as obesity and diabetes.

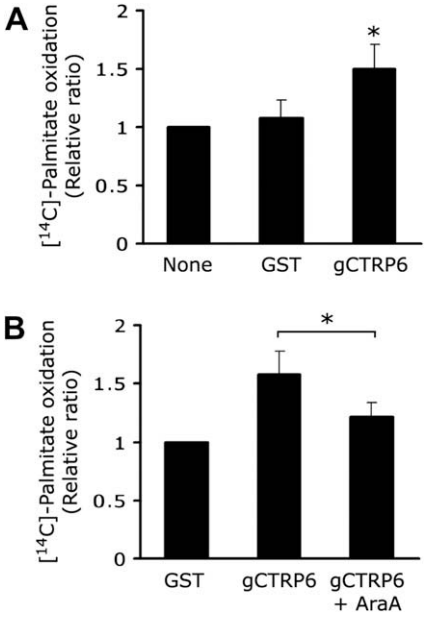

Fig. 4. Effect of gCTRP6 on fatty acid oxidation in C2C12 myotubes. (A) C2C12 myotubes were starved of serum for 2 h and washed twice with HEPES-buffered saline solution. The cells were treated with GST (10 μg/ml) or GST-gCTRP6 (10 μg/ml) for 45 min. The fatty acid oxidation rate was measured as $^{14}CO_2$ generation from ^{14}C-palmitate. The trapped $^{14}CO_2$ and ^{14}C-labeled acid soluble products were determined by liquid scintillation counting and expressed as a relative ratio, where the radioactivity of untreated control cells was set to one. Results are expressed as the mean ± S.D. of three independent experiments. AVOVA: $^{*}P < 0.05$. (B) C2C12 myotubes were treated with GST-gCTRP6 (10 μg/ml), with or without 1-h pretreatment of AraA (2 mM). The fatty acid oxidation was measured as $^{14}CO_2$ generation from ^{14}C-palmitate. The trapped $^{14}CO_2$ and ^{14}C-labeled acid soluble products were determined by liquid scintillation counting and expressed as a relative ratio, where the radioactivity of GST-treated cells was set to one. Results are expressed as the mean ± S.D. of three independent experiments. *t*-test: $^{*}P < 0.05$.

Acknowledgments

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